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Nitrogen Removal by Tubular Gel Containing *Nitrosomonas europaea* and *Paracoccus denitrificans*

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A new bioreactor for the removal of nitrogen from wastewater is described which consists of a tubular polymeric gel containing *Nitrosomonas europaea* and *Paracoccus denitrificans*. The outer surface of the tube is in aerobic contact with wastewater containing ammonia, while the inside of the tube is in anaerobic contact with ethanol flowing through the tube. *N. europaea* oxidizes ammonia to nitrite in the gel, and then *P. denitrificans* reduces the nitrite to nitrogen gas in the same gel. This concept would be effective for simplifying nitrogen removal systems requiring aerobic and anaerobic operations.

Eutrophication in many lakes and coasts is an environmental problem which has been accelerated by the excessive nitrogen content originating in domestic and industrial effluent (14). There is great interest in the development of an effective wastewater treatment system for nitrogen removal. The presently available systems for nitrogen removal use two biological conversion steps, aerobic nitrification and anaerobic denitrification (22). If nitrification and denitrification occurred in the same unit, nitrogen removal systems would be simplified. It has been reported that nitrification and denitrification occur simultaneously in submerged soils (15) and sediments (3, 8, 17) in the natural ecosystem and in an artificial ecosystem composed of polymeric beads (11, 12, 18). However, it is difficult to maintain an artificial condition which is suitable for both nitrification and denitrification. Here, we discuss a new bioreactor consisting of a tubular gel containing an ammonia oxidizer, *Nitrosomonas europaea*, and a denitrifier, *Paracoccus denitrificans.*

Immobilization method. *N. europaea* IFO-14298 was grown in Soriano-Walker medium (20), and *P. denitrificans* JCM-6892 was cultured in nutrient broth (Difco) with incubation at 30°C. Each strain was harvested by centrifugation (20,000 × *g*, 10 min, 4°C) and washed three times with a phosphate buffer (9 g of Na_2HPO_4 per liter, 1.5 g of KH_2PO_4 per liter [pH 7.5]). Eight milligrams (dry weight) of *N. europaea* alone or 8 mg (dry weight) of *N. europaea* and 66 mg (dry weight) of *P. denitrificans* were suspended in 3 ml of the phosphate buffer. The suspensions were mixed with 9 ml of photo-cross-linkable polymer PVA-SbQ (SPP-H-13, Toyo Gosei Kogyo Corp.). They were formed into a plate (50-mm diameter, 5 mm thick) or a tube (12-mm outside diameter, 5-mm inside diameter, 125 mm long by using a culture dish or a glass tube as a mold, and they were solidified into a gel by metal halide light irradiation (1,000 μmol m^{-2} s^{-1} of light intensity) for 1 h. To avoid photoinactivation during the immobilization (5), the gels were kept at 0°C in molds without being exposed to air. Silicon tubes (4-mm outside diameter, 2-mm inside diameter) were attached to both sides of the tubular gel to circulate ethanol solution.

Wastewater treatment. Artificial wastewater (200 ml) was treated at 30°C with aeration (100 ml/min) and stirring (300 rpm) by the plate gel (Fig. 1A) or tubular gel system (Fig. 1B). The wastewater contained (per liter) 0.944 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 9 g of Na_2HPO_4, 1.5 g of KH_2PO_4, and 1 ml of a solution containing trace elements (2). The pH in the wastewater was adjusted to 7.8 by adding 1 M K_2CO_3. Ethanol was used as an electron donor for denitrification. When the plate gel containing *N. europaea* and *P. denitrificans* was used, 0.125 ml of 99.5% ethanol was injected every 24 h into the space between the gel and the culture dish. When the tubular gel was used, 10 ml of the phosphate buffer containing 2.0% (vol/vol) ethanol was circulated through the inside of the tube (rate of flow, 5 ml/h). The ethanol solution was purged from air by N_2 gas. Ammonia and nitrite concentrations in the wastewater were colorimetrically measured according to previously reported methods (4). Nitrate concentration was determined by using an ion-chromato analyzer (IC-500P; Yokogawa Electric Corp.) with a SAX1-205 column. Gas produced in the gel was analyzed by a gas-chromato analyzer (G-3000; Hitachi Corp.) with a unibeads C60/80 mesh column and a photo ionization detector.

Fluorescent-antibody labeling. The tubular gel was sampled at the start, the fourth day, and the seventh day of treatment. These gels were fixed, dehydrated, and embedded in polyethylene glycol by the procedure described by Hunik et al. (6). The sections were labeled with rabbit anti-*N. europaea*-fluorescein isothiocyanate and rabbit anti-*P. denitrificans*-fluorescein isothiocyanate for 45 min at 52°C in the dark. These specific antibodies were made by a previously reported method (19). Each antibody stained each strain specifically and showed no cross-reaction. The stained sections were observed by using a fluorescent microscope (AHBS3; Olympus Corp.) with a DPlanApo UV objective (40×, 1.00 numerical aperture), a DM505 dichroic mirror, a 20BP495 excitation filter, and an 18O515IF emission filter. The obtained photomicrographs were converted into distribution densities by image analysis (IPLab Spectrum, Signal Analytics Corp.).

Ammonia removal by plate gel. The artificial wastewater was aerobically treated by plate gels which contained either both *N. europaea* and *P. denitrificans* or *N. europaea* alone in batch systems (Fig. 1A). When treated by the gel containing *N. europaea* alone, the ammonia concentration in the wastewater decreased with the increase of the nitrite concentration (Fig. 2A). The sum of the ammonia and nitrite concentrations was constant. On the other hand, when treated by the gel containing both *N. europaea* and *P. denitrificans*, the ammonia concentration decreased without an increase of the nitrite concentration (Fig. 2B). Nitrate was not detected (<0.05 mg of N per

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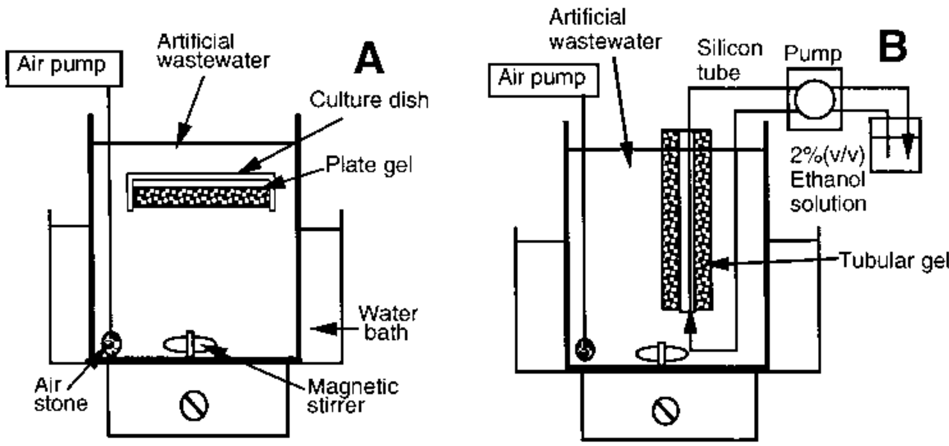

FIG. 1. Schematic diagrams of experimental apparatus with plate gel (A) and tubular gel (B) in batch system.

liter) in the treated water during the experiment. These results suggest that nitrification and denitrification occur simultaneously in the gel. *N. europaea* requires aerobic conditions to oxidize ammonia to nitrite (24, 25), while *P. denitrificans* requires strict anaerobic conditions for reducing the nitrite to nitrogen gas (9, 10). The gel provides both aerobic and anaerobic regions.

The nitrification (oxidation of ammonia to nitrite) rate of the plate gel was calculated based on changes in ammonia and nitrite concentrations over the initial 13.5 h of the experiments (shown in Fig. 2A and B). The nitrification rate of the gel containing *N. europaea* and *P. denitrificans* was 0.263 $\pm$ 0.011 (mean $\pm$ standard deviation, n = 3) g of N per h for 1 m^2 of gel surface, while that of the gel containing *N. europaea* alone was 0.093 $\pm$ 0.016 g of N per h. The coimmobilized bacteria accelerated the nitrification rate three times faster than the rate of the immobilized ammonia oxidizer alone, although the initial densities of *N. europaea* in the two gels were the same. This result suggests that *N. europaea* and *P. denitrificans* work synergetically.

Ammonia removal by tubular gel. The artificial wastewater was aerobically treated by the tubular gel containing *N. europaea* and *P. denitrificans* in a batch system (Fig. 1B). The ammonia concentration in the wastewater decreased without an increase in the nitrite concentration (Fig. 2C). Nitrate was not detected in the treated water. The nitrification rate, which was calculated on the basis of the ammonia and nitrite concentrations for the initial 13.5 h, was 0.246 g of N per h for 1 m^2 of gel surface. It was 10% less than the rate of the plate gel containing both strains for the initial 13.5 h, shown in Fig. 2B. Nitrous oxide was not detected (<1 ppm) in the produced gases.

In the nitrogen removal system with coimmobilized polymer beads, an excessive electron donor for denitrification has to be directly added to the wastewater, and then the residual electron donor is removed from the treated water by aeration (11, 12, 18). The direct addition of an electron donor to the wastewater results in an increase of heterotroph. The increase of heterotroph results in not only the waste of the electron donor for denitrification but also the waste of the oxygen for ammo-

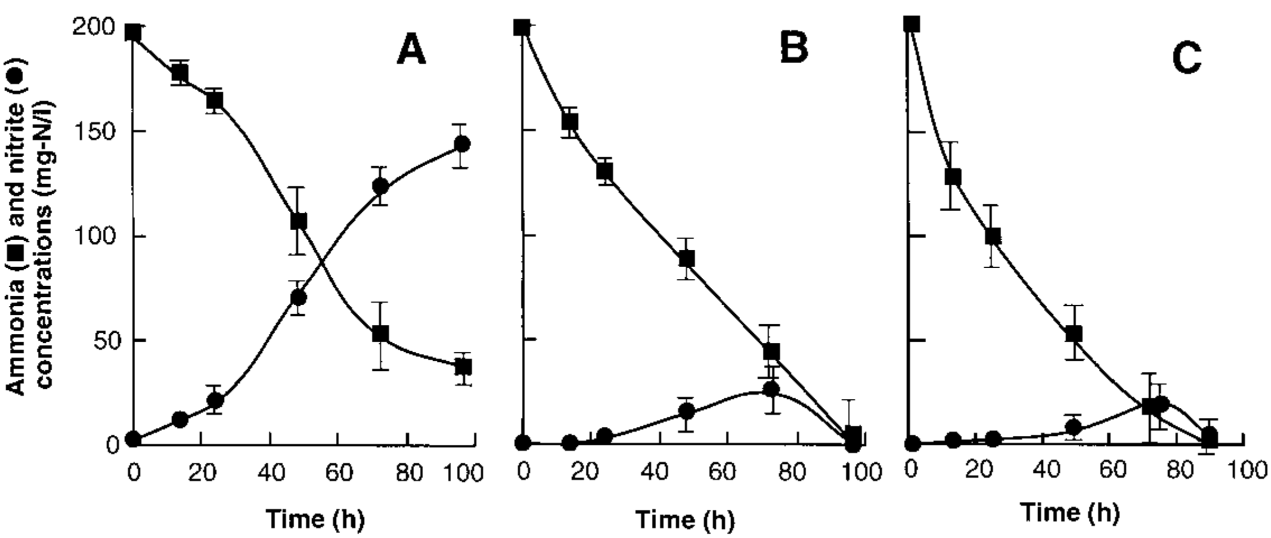

FIG. 2. Changes in ammonia and nitrite concentrations (mg of N per liter) in wastewater. The wastewater was treated by the plate gels and the tubular gel. The plate gel contains *N. europaea* (A) or *N. europaea* and *P. denitrificans* (B). The tubular gel contains *N. europaea* and *P. denitrificans* (C). Bars show standard deviations (n = 3).

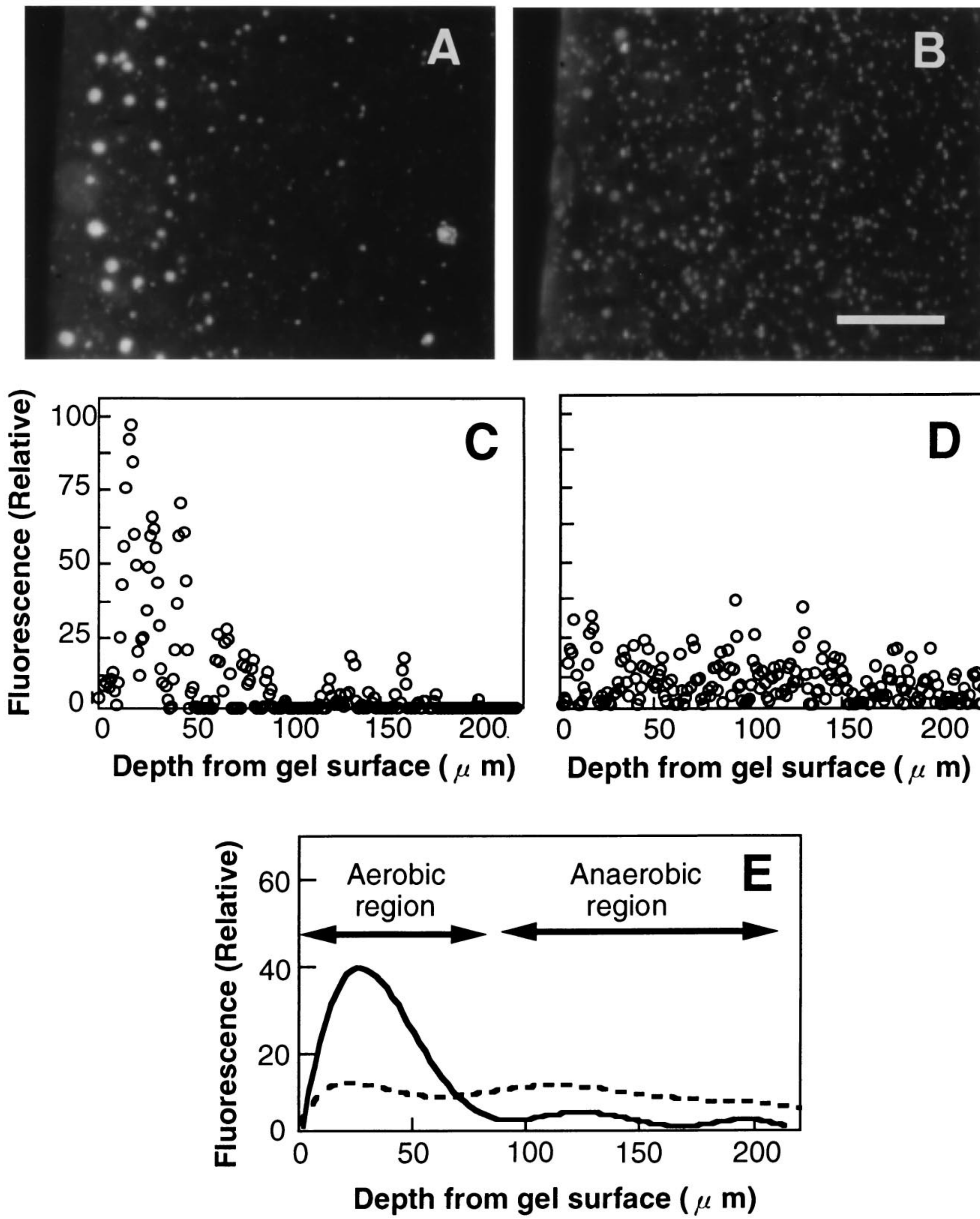

FIG. 3. Distributions of *N. europaea* and *P. denitrificans* in tubular gel. Fluorescent photomicrographs of *N. europaea* (A) and *P. denitrificans* (B) in cross sections of the tubular gel. The left side of the photographs shows the outer surface of the gel. Bar = 50 μm. Fluorescent distributions (C and D) were determined by image analysis based on the photomicrographs (panels A and B), respectively. The solid and dotted curves (E) show the relative biomass of *N. europaea* and *P. denitrificans*, respectively. These curves were constructed from the data in panels C and D by an eighth-order polynomial.

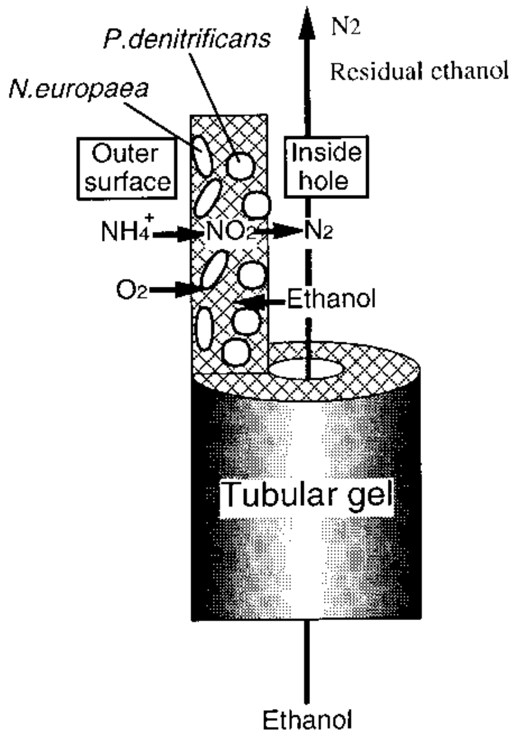

FIG. 4. Nitrogen removal by using tubular polymeric gel.

nia oxidation. Nitrogen removal under low oxygen pressure is unfavorable for ammonia oxidation. For such a condition, nitrous oxide, green effect gas, may be produced by the ammonia oxidizers (1, 7, 16, 21) and denitrifiers (1, 13, 23). It is not easy with the coimmobilized beads to control the conditions for denitrification. In this study, ethanol was supplied to *P. denitrificans* in the tubular gel by circulating it through the tube without mixing it into the wastewater. Therefore, the bioreactor with the tubular gel not only can omit the step of removing the residual electron donor but also can provide favorable conditions for ammonia oxidation and denitrification.

Distributions of *N. europaea* and *P. denitrificans* in the tubular gel. Distributions of *N. europaea* and *P. denitrificans* in the tubular gel were microscopically investigated by fluorescent-antibody labeling techniques. Each distribution was determined at the start, the fourth day, and the seventh day of treatment. Figure 3A and B shows photomicrographs of the cross sections of the tubular gel at the seventh day. Fig. 3C and D shows the fluorescent distributions determined by image analysis based on the photomicrographs. Fig. 3E was constructed from Fig. 3C and D by an eighth-order polynomial. The colonies of *N. europaea* were mostly concentrated at a depth of 20 μm from the outer surface. These colonies were larger than the others. On the other hand, the colonies of *P. denitrificans* were spread equally throughout the gel. Few large colonies of *P. denitrificans* were observed in the gel. Tiny colonies of *N. europaea* were dispersed in the gel at the start. The colonies close to the surface of the gel grew to be larger colonies during the 7-day treatment, while those in the middle of the gel remained at their initial size. The distribution of *N. europaea* at the fourth day had the same characteristics as that at the seventh day, as shown in Fig. 3A. On the other hand, the distribution and colony size of *P. denitrificans* in the gel were almost the same at the start, the fourth day, and the seventh day.

The growth of *P. denitrificans* in the gel is not restricted by oxygen supply, since *P. denitrificans* can use either oxygen or nitrite as an electron acceptor for respiration (9, 10). On the other hand, the growth of *N. europaea* is restricted by oxygen and ammonia supply (24, 25). In this study, its growth is restricted by oxygen supply rather than by ammonia, since the ammonia could go through the gel, which process was detected in the ethanol solution. *N. europaea* grew and consumed oxygen and then produced the anaerobic region. The aerobic region for nitrification was located close to the outer surface of the gel, and the anaerobic region for denitrification was inside the gel. The borderline between the aerobic region and the anaerobic region was at a depth of about 80 μm from the gel surface, where *N. europaea* drops off (Fig. 3E).

Here, we have shown a novel concept for nitrogen removal. In the gel, *N. europaea* oxidizes ammonia to nitrite, and then *P. denitrificans* reduces the nitrite to nitrogen gas (Fig. 4). Ammonia is converted into nitrogen gas directly and removed from the wastewater rapidly. Ethanol as an electron donor for denitrification can be used by circulating it through the inside of the tube, without mixing it into the wastewater directly. We expect that a bioreactor with the tubular gel could be applied in various practical systems, since the concept, as shown in this paper, is simple and effective.

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